The glutathione peroxidase gene family of *Lotus japonicus.* Characterization of genomic clones, expression analyses and immunolocalization in legumes

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Summary

● Glutathione peroxidases (GPXs) catalyze the reduction of H_2O_2 and phospholipid hydroperoxides using preferentially thioredoxins as electron donors. We have characterized six *GPX* genes, including promoters, of the model legume *Lotus japonicus*.

● Gene expression was analyzed by quantitative reverse transcription (qRT)-PCR in leaves, roots and nodules of *L. japonicus* plants at the late vegetative growth stage. In addition, *L. japonicus* and *L. corniculatus* plants were exposed for 1 to 24 h to various treatments known to generate reactive oxygen and/or nitrogen species to assess their effects on *GPX* expression in roots.

● *LjGPX1* and *LjGPX3* are the most abundantly expressed genes in leaves, roots and nodules. Compared with roots, *LjGPX1* and *LjGPX6* are highly expressed in leaves and *LjGPX3* and *LjGPX6* in nodules. Concerning stress conditions, salinity (150 mM NaCl) had no significant effect except for a decrease (<0.5-fold) in *GPX4* expression; aluminum (20 μM) decreased expression of the six genes; and cadmium (20 μM) caused up-regulation (2.7-fold) of *GPX3*, *GPX4* and *GPX5* after 1 h and down-regulation of *GPX1*, *GPX2*, *GPX4* and *GPX6* after 3-24 h. However, the major effects were observed with sodium nitroprusside, a nitric oxide (NO) donor. After only 1 h, the mRNA levels of *GPX3*, *GPX4* and *GPX6* were increased by 2.5-, 4.3- and 37-fold, respectively. After 3, 6 and 24 h, the *GPX6* mRNA levels remained 17-, 3- and 9-fold over the control, respectively. Immunogold labelling revealed the presence of GPX proteins in root and nodule amyloplasts and in leaf chloroplasts of *L. japonicus* and other legumes. Labelling was specifically associated with starch grains.

● Our results underscore the differential regulation of *GPX* expression in response to cadmium, aluminum and NO, and strongly support a role for *GPX6* and possibly other *GPX* genes in stress and/or metabolic signalling.

Key words*:* Glutathione peroxidases, metal toxicity, model legumes, nitric oxide, plastids, salt stress, starch.

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Introduction

In plants, reactive oxygen species (ROS), such as the superoxide radical and H_2O_2, are formed mainly in the chloroplasts, mitochondria, peroxisomes and apoplast (del Río *et al.*, 2002; Mittler, 2002). These ROS are potentially toxic when produced at high rates and can give rise to highly oxidizing hydroxyl radicals through Fenton reactions (Halliwell & Gutteridge, 2007). Similarly, reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite ($ONOO^-$), are produced in different subcellular compartments of plants (del Río et al., 2002; Lamattina *et al.*, 2003; Valderrama *et al.*, 2007). The reaction between NO and superoxide or between nitrite and H_2O_2 can generate peroxynitrite, which causes oxidation and nitration of proteins and DNA (Halliwell & Gutteridge, 2007). However, under controlled, low steady-state concentrations, ROS and RNS fulfil essential functions in growth and development and in redox signalling during the stress responses of plants (Mittler, 2002; Lamattina *et al.*, 2003; Foyer & Noctor, 2005). To keep cellular levels of ROS and RNS under tight control, plants contain antioxidant enzymes and metabolites at variable amounts in different tissues, cells and organelles.

Glutathione peroxidases (GPXs) catalyze the reduction of lipid peroxides and other organic peroxides to the corresponding alcohols using thioredoxins as the preferred electron donors (Herbette *et al.*, 2002; Navrot *et al.*, 2006). In mammals, there exist five distinct groups of GPXs that differ in structure, substrate specificity and subcellular localization (Ursini *et al.*, 1995). Considerably less is known about plant GPXs. They are most similar in terms of amino acid sequences to the mammalian GPX4 type of enzymes, which comprises the selenium-dependent phospholipid hydroperoxide GPXs. Recently, a GPX from the green alga *Chlamydomonas reinhardtii* has been characterized and shown to contain a selenocysteine residue at the catalytic site (Fu *et al.*, 2002), but all GPXs from vascular plants so far identified have a cysteine residue instead (Navrot *et al.*, 2006). The major function of GPXs in plants appears to be the scavenging of phospholipid hydroperoxides and thereby the protection of cell membranes from peroxidative damage (Gueta-Dahan *et al.*, 1997). Recent data showed that some GPXs may be involved also in redox transduction under stressful

conditions (Miao *et al.*, 2006). Consistent with these two functions, the expression of many GPXs is enhanced in response to abiotic and biotic stresses, including salinity, heavy metal toxicity and infection with bacterial or viral pathogens (Avsian-Kretchmer *et al.*, 2004; and references therein).

Several cDNA clones that encode GPXs have been isolated from diverse plants of agronomic interest, such as citrus (*Citrus sinensis*; Holland *et al.*, 1993), pea (*Pisum sativum*; Mullineaux *et al.*, 1998), barley (*Hordeum vulgare*; Churin *et al.*, 1999) and tomato (*Lycopersicon esculentum*; Herbette *et al.*, 2002). However, up to date, the *GPX* genes have been studied comprehensively only in two plant species, thale cress (*Arabidopsis thaliana*; Rodriguez Milla *et al.*, 2003) and poplar (*Populus trichocarpa*; Navrot *et al.*, 2006). Abundant information in the databases is also available for rice (*Oryza sativa)*, whose genome has been completely sequenced. Comparable studies of the *GPX* genes have not been performed for any leguminous plant, despite the multiple roles that antioxidants play in the rhizobia-legume symbiosis (Dalton, 1995; Matamoros *et al.*, 2003; Puppo *et al.*, 2005). Two legume species, *Lotus japonicus* and *Medicago truncatula*, are currently used as models for classical and molecular genetics. In this work, we have identified six *GPX* genes in *L. japonicus*, determined their complete structures and promoter sequences, and quantified their expression levels in leaves, roots and nodules. To gain further insights into the regulation of *LjGPX* genes, plants of *L. japonicus* and birdsfoot trefoil (*Lotus corniculatus*), a related species of agronomic interest, were subjected to treatments known to generate ROS (Sugimoto & Sakamoto, 1997; Avsian-Kretchmer *et al.*, 2004; Romero-Puertas *et al.*, 2004; Sharma & Dubey, 2007) and/or RNS (Bethke *et al.*, 2006; Valderrama *et al.*, 2007). Thus, *GPX* gene expression was investigated in roots of plants treated with salt (NaCl), cadmium (Cd), aluminum (Al) or the NO donor sodium nitroprusside (SNP). Finally, we have immunolocalized the GPX protein(s) in *L. japonicus* and other legumes.

Materials and Methods

Biological material and plant treatments

For studies with nodulated plants (expression analyses in leaves, roots and nodules), seeds of *Lotus japonicus* cv. MG20 were scarified, surface disinfected and germinated in petri dishes on filter paper for 2 d at 4˚C, 1 day at 22˚C in the dark and 2 d at 22˚C in the light. Seedlings were then transferred to vermiculite-containing pots, nodulated with *Mesorhizobium loti* strain R7A and grown under controlled environment conditions [24˚C/18˚C (day/night), 180 μmol $m^{-2}s^{-1}$, 16-h photoperiod]. Plants were irrigated once a week with B&D nutrient solution (Broughton & Dilworth, 1971), supplemented with 0.25 mM NH_4NO_3, until they were 42 d old (Rubio *et al.,* 2007).

For studies with non-nodulated plants (expression analyses in roots of stressed plants), we found that growth of plants in hydroponic cultures were critical for a strict control of the very short treatments. To this end, seeds of *L. japonicus* cv. MG20 and *L. corniculatus* cv. Draco were germinated in petri dishes as indicated above, and seedlings were transferred to 10 x 10 cm plates (8 seedlings per plate) containing 50 mL of a modified Fahraeus medium (Boisson-Dernier *et al.,* 2001) with 15 g L^{-1} of agar for 7 d (same controlled conditions as before) to allow adequate root growth. Finally, plants were transferred to hydroponic solutions, grown under the same controlled conditions as the nodulated plants (except that NH_4NO_3 concentration was 1.25 mM) and treated as follows: (a) *L. corniculatus* plants were grown on solution A (water plus 200 μM $CaCl_2$, at pH 6.0) for 7 d and were treated with 20 μM $CdCl_2$ for 1-24 h; (b) *L. corniculatus* plants were grown on solution B (water plus 200 μM $CaCl_2$, at pH 4.0) for 7 d and were treated with 20 μM $AlCl_3$ for 1-24 h; and (c) *L. japonicus* plants were grown on solution C (one-fourth strenght B&D nutrient solution, at pH 6.0) for 10 d and were treated with 150 mM NaCl, 0.1 mM SNP or 0.1 mM potassium ferricyanide for 1-24 h. Solutions were continuously aerated and replaced every 2 d. We used solutions A and B to avoid interactions of the nutrient salts with Cd and Al, and the pH of solution B was adjusted to 4.0 to avoid Al precipitation (Sugimoto & Sakamoto, 1997). Plants

had no apparent symptoms of stress by metals or acid pH, with the exception of an increased proliferation of lateral roots at low pH.

For immunolocalization studies, two additional legume species were included. Alfalfa (*Medicago sativa* cv. Aragón), which produces indeterminate root nodules, and *Sesbania rostrata*, which produces both root and stem (photosynthetically active) nodules, were grown under controlled conditions as described by Rubio *et al.* (2004) and James *et al.* (1996), respectively. Nodules from these two legumes were harvested 30-35 d after inoculation.

Plant material to be used for determination of mRNA or protein levels was flash-frozen in liquid nitrogen and stored at -80ºC. Plant material to be used for electron microscopy (EM) studies was harvested fresh and immediately immersed in 2.5% glutaraldehyde (for details see further below).

Identification, mapping, and promoters of *LjGPX* genes

The transformation competent artificial chromosome (TAC) clones, LjT04E19 (*LjGPX1* and *LjGPX2*), LjT13O11 (*LjGPX3*), LjT08L06 (*LjGPX4*), LjT23J20 (*LjGPX5*) and LjT10B19 (*LjGPX6*), were isolated by screening TAC and bacterial artificial chromosome (BAC) genomic libraries using expressed sequence tag (EST) or tentative consensus (TC) sequences. The accession numbers of these sequences, as well as those of the genomic clones, are listed in Table 1. The nucleotide sequences of the candidate TAC clones were determined according to the bridging shotgun method (Sato *et al.*, 2001).

The *LjGPX* genes were mapped using simple sequence repeat markers indicated in Table 1. These markers were used for genotyping of the F_2 mapping population of the B-129 x MG-20 cross, as described (Sato *et al.*, 2001). The *LjGPX6* gene was mapped using a simple sequence repeat marker found in the TAC clone LjT25H19, which overlapped with LjT10B19. A primer set (5'-GCTTTCACTTTTCTAATTGAAAAT-3' and 5'-AAGCA CATATTCTTGCCTTC-3') that amplified 142 bp and 146 bp products from *L. japonicus* cvs. B-129 and MG-20, respectively, was used for genotyping of the mapping population. The

promoter regions of the six *LjGPX g*enes were analyzed *in silico* for the presence of *cis*-acting regulatory elements using the PlantCARE database algorithm (Lescot *et al.,* 2002).

Predicted properties and subcellular localization of LjGPX proteins

The deduced sequences of the LjGPXs were aligned with the PileUp program to identify conserved domains and were used, together with the sequences of the homolog proteins from other vascular plants, to construct an unrooted phylogenetic tree by the neighbor-joining method with CLUSTALW 1.75 (Thompson *et al.,* 1994) and TreeView (Page, 1996) programs. Predictions of subcellular localization were performed with the programs TargetP 1.1 (Emanuelsson *et al.,* 2000), MitoProtII 1.0a4 (Claros & Vincens, 1996) and PSORT (Nakai & Kanehisa, 1992).

Expression analysis of *LjGPX* genes

Total RNA was isolated from leaves, roots or nodules using the RNAqueous kit (Ambion, Cambridgeshire, UK). The RNA was treated with DNaseI (Roche, Penzberg, Germany) to remove traces of genomic DNA, and reverse transcription was performed using poly-T_{17} primer and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Real-time qRT-PCR analysis was carried out with the iCycler iQ System (Bio-Rad, Hercules, CA, USA) using the iQ SYBR-Green Supermix (Bio-Rad) and gene-specific primers (Supplementary Material Table S1). The PCR program consisted of an initial denaturation and *Taq* activation step of 95ºC for 5 min, followed by 50 cycles of 95ºC for 15 s and 60ºC for 1 min. The specificity of primers and the absence of contaminating genomic DNA were verified by amplicon dissociation curves and gel-electrophoretic analysis. An additional control, consisting of a PCR analysis of RNA samples prior to reverse transcription, was also included to discard any contamination with genomic DNA. The amplification efficiency of primers, calculated by serial dilutions of root and leaf cDNAs, was >80%. The mRNA levels were normalized with *ubiquitin* as the reference gene and were expressed using the method of Livak & Shmittgen (2001). All the reactions were set up in

duplicate (two technical replicates) using three to five RNA preparations (biological replicates) from different plants. The threshold cycle (C_T) values were in the range of 19-21 cycles for *ubiquitin* and 21-30 cycles for the *LjGPX* genes.

Immunoblot analysis and immunolocalization of GPX proteins

For immunoblots, proteins were extracted from roots and leaves at 0ºC with 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100, and were quantified by the Bradford dye-binding microassay (Bio-Rad). Proteins were resolved in 15% SDS-gels and were transferred onto polyvinylidene fluoride membranes (Pall Corporation, Ann Arbor, MI, USA) using a transfer buffer consisting of 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol. Equal loading of lanes and transfer quality were verified by Ponceau staining of membranes. Immunoblot analyses were performed using an affinity-purified polyclonal antibody raised against poplar GPX3.2 (Navrot *et al.*, 2006) at a dilution of 1:1,000. Goat anti-rabbit horseradish peroxidase conjugated antibody (Sigma-Aldrich) was used as the secondary antibody at a dilution of 1:20,000, with 5% (w/v) nonfat milk to reduce background signal. Immunoreactive proteins were visualized using the SuperSignal West Pico (Pierce) chemiluminescent reagent for peroxidase detection.

For EM studies, nodules of *L. japonicus* were fixed in 2.5 % glutaraldehyde and then high pressure frozen and embedded in low temperature resin (Lowicryl HM23; Polysciences, Warrington, USA) according to Moran *et al.* (2003). Unfixed root nodules, stem nodules and leaves from *S. rostrata* were prepared in the same way. Roots and leaves of *L. japonicus* and nodules of alfalfa (*M. sativa*) were fixed in 2.5% glutaraldehyde and then embedded in LR White acrylic resin (Agar Scientific, Stansted, UK) as previously described for nodules of *L. corniculatus* and *L. uliginosus* by James & Sprent (1999). Immunogold labelling of ultrathin sections was performed according to Moran *et al.* (2003) using a 1:10 dilution of the GPX3.2 antibody and a 1:100 dilution of the secondary goat anti-rabbit antibody conjugated to 15-nm gold particles (GE Healthcare, Little Chalfont, UK). Serial sections incubated in non-immune serum, which was purified with protein A and diluted 1:10, were used as a negative control.

The sections were viewed and photographed using a JEOL 1200 EX transmission electron microscope.

Results and Discussion

Identification and characterization of *LjGPX* genes

The first aim of this study was to isolate and characterize the *GPX* genes of *L. japonicus*. A search in the gene databases of this model legume allowed us to identify three TC sequences that contained the complete open reading frames (ORFs), as well as some 5'- and 3'-untranslated (UTR) sequences, of *LjGPX1*, *LjGPX3* and *LjGPX6* (Table 1). Database mining also resulted in the identification of two singletons that were assigned to *LjGPX4* and *LjGPX5*. An additional gene, *LjGPX2*, was identified by screening the TAC libraries using the TC sequence of *LjGPX1*. The *LjGPX2* gene was found to be transcribed by determination of mRNA levels, as there were no ESTs available for this gene. In fact, the number of ESTs for each gene (Table 1), which were obtained by reverse transcription of total RNA from young plants, inmature flowers, pods, roots and nodules of *L. japonicus*, strongly suggests that the expression of *LjGPX2*, *LjGPX4* and *LjGPX5* is much lower than that of *LjGPX1* and *LjGPX3*. The EST sequence information was also used to isolate TAC clones and to map five of the genes on chromosomes 4 and 5 (Table 1). Interestingly, *LjGPX1* and *LjGPX2* are tandemly arranged with the same orientation on the chromosome, and their ORFs are separated by only 1477 bp. However, these two genes show only 71% identity in their ORFs, indicating that they did not originate from a recent duplication event.

To establish the exon-intron organization of the less expressed *LjGPX* genes, namely, *LjGPX2, LjGPX4* and *LjGPX5*, their ORFs were entirely sequenced using information based on the *L. japonicus* genome project (Sato *et al.*, 2001). All the genes, except *LjGPX4*, contain six exons interrupted by five introns (Fig. 1). The same exon composition was observed for the eight *GPX* genes of *A. thaliana* (Rodríguez Milla *et al.*, 2003), the six *GPX* genes of poplar (Navrot *et al.*, 2006) and the single *GPX* gene of citrus (*CsGPX1*) so far examined

(Avsian-Kretchmer *et al.,* 2004). Thus, all the *AtGPX* and *PtGPX* genes have six exons and a previous annotation (Rodríguez Milla *et al.,* 2003) that *AtGPX3* and *AtGPX7* have seven and five exons, respectively, was found to be incorrect. There is also a high conservation among plant species in the exon lengths. Exons 2 to 5 have identical size for all the *LjGPX* and *AtGPX* genes, with the exception of exon 5 in *LjGPX4*. The length of exon 1 in the *LjGPX*, *AtGPX*, *OsGPX* and *PtGPX* genes is variable, as expected for genes encoding GPX isoforms lacking or bearing N-terminal signal peptides. Exon 6 is absent in *LjGPX4* and has a similar size in the other *LjGPX* genes (30 to 45 bp) or in the *AtGPX* genes (30 to 39 bp).

The promoter regions of the six *LjGPX* genes were searched for the presence of putative *cis*-acting regulatory elements as an indication of the responsiveness of the genes to hormones or environmental cues. Most or all the *LjGPX* promoters contain elements that are responsive to light, biotic stress and abiotic stress, whereas only some of them contain elements that are involved in the response to anaerobiosis and hormones (Supplementary Material Table S2). A similar conclusion was drawn from the *in silico* analysis of the *AtGPX* promoters (Rodríguez Milla *et al.,* 2003). The *LjGPX1* and *LjGPX6* promoters also contain *cis*-sequences, with one or two mismatches, that may be responsive to oxidative stress. These sequences include the antioxidant responsive element (ARE), which also has been found in the promoters of the three *catalase* genes of maize (Scandalios *et al.,* 1997).

Predicted properties of LjGPX proteins

The derived amino acid sequences of the six LjGPX proteins contain the three motifs present in most plant and mammalian GPXs (Criqui *et al.,* 1992; Depège *et al.,* 1998; Churin *et al.,* 1999). These motifs contain residues that are proposed to be part of the catalytic site (LjGPX1 numbering): Cys-111, Glu-112, Cys-140, Gln-142 and Trp-200. The presence of Cys-111 indicates that none of the six LjGPX proteins is a selenium-dependent enzyme, as this amino acid residue is replaced by selenocysteine in the homolog proteins of mammals (Ursini *et al.,* 1995) and *C. reinhardtii* (Fu *et al.,* 2002). From the alignment of LjGPX sequences of Figure 2 and the data presented in Table 2, three types of proteins have been differentiated based on

the amino acid length: LjGPX1 and LjGPX6 (*c.* 235 amino acids, 26 kD); LjGPX3 (211 amino acids, 24 kD); and LjGPX2, LjGPX4 and LjGPX5 (*c.* 170 amino acids, 19 kD). These differences, and the poor homology among the first 40 or 70 amino acid residues of LjGPX1, LjGPX3 and LjGPX6 (Fig. 2), clearly suggest that these three proteins bear signal peptides for organelle targeting. Prediction programs of subcellular localization suggest that LjGPX6 has a chloroplastic N-terminal transit peptide and that LjGPX1 has an ambiguous N-terminal peptide for targeting to mitochondria and plastids. The same programs predicted that LjGPX2, LjGPX4 and LjGPX5 are localized in the cytosol, and LjGPX3 in the cytosol and secretory pathway (Table 2).

The full-length amino acid sequences of plant GPXs available in the databases were used to build an unrooted phylogenetic tree (Fig. 3). Inclusion of the complete sequences rather than mature sequences was preferred to construct the tree because there was uncertainty about the cleavage sites for many of the proteins. Alignment of the whole sequences by CLUSTALW was consistent with the homologies among the predicted mature proteins. The putative subcellular localizations are marked with different colors in the same figure. The tree is composed of five clades and a separated branch for barley GPX3. Clades I and II are hypothesized to contain, respectively, chloroplastic and cytosolic isoforms; clades III and IV, both cytosolic and secreted proteins; and clade V, cytosolic proteins and proteins with N-terminal transit peptides for targeting either to the mitochondria or to both the mitochondria and chloroplasts. This phylogenetic analysis updates a previous version and is fully consistent with the assignments made for clades I and II (Navrot *et al.*, 2006). However, careful inspection of amino acid sequences by us and others (Rodríguez Milla *et al.*, 2003) strongly suggests that at least some proteins of clades III and V may be targeted to multiple subcellular compartments. This hypothesis is based on the presence of ambiguous N-terminal signal peptides in LjGPX1 or of putative alternative translation sites in the mRNAs of LjGPX1, LjGPX3 and AtGPX6 (Table 2; Rodríguez Milla *et al.*, 2003).

Expression analyses of *LjGPX* genes in plant organs

The steady-state mRNA levels of the *LjGPX* genes were determined by qRT-PCR in leaves, roots and nodules of *L. japonicus.* This study allowed us to compare the abundance of the six *LjGPX* mRNAs within each plant organ (Fig. 4a) as well as the abundance of a specific *LjGPX* mRNA among the different plant organs (Fig. 4b). The first comparison was made by normalizing all mRNA levels with respect to those of one gene with significant expression, such as *LjGPX6*. It can be clearly observed that *LjGPX1* and *LjGPX3* are the most abundantly expressed genes in all three organs. Relative to *LjGPX6*, the mRNA level of *LjGPX1* was 6-fold greater in the leaves and nodules and 25-fold greater in the roots; also, the mRNA levels of *LjGPX3* were 6-, 30- and 43-fold greater, respectively, in the leaves, roots and nodules. On the contrary, the less abundant transcripts (<0.5-fold relative to *LjGPX6*) were those of *LjGPX2* and *LjGPX5* in the leaves and those of *LjGPX4* in all three plant organs (Fig. 4a). The second comparison was made by normalizing the mRNA levels with respect to those found in the roots (Fig. 4b). It can be seen that there was high expression levels of *LjGPX6* (4-fold) in leaves and of *LjGPX3* (2.5-fold) and *LjGPX6* (3-fold) in nodules. In contrast, *LjGPX4* expression was negligible (<0.01-fold) in both leaves and nodules (Fig 4b). Interestingly, a consistent up-regulation (6.8-fold) of the *LjGPX3* gene in nodules with respect to uninfected roots was also detected in a transcriptomic study using cDNA arrays (Colebatch *et al.*, 2002).

Expression analyses of *GPX* genes in plants exposed to stressful conditions

The expression at the transcriptional level of the *GPX* genes was studied in *L. japonicus* and *L. corniculatus* plants exposed to several stress treatments involving production of ROS and/or RNS. We included *L. corniculatus* in this expression analysis to extend the molecular information gained with *L. japonicus* to other legume species, as we confirmed by melting curve analysis of the PCR products that the gene-specific primers were also valid for *L. corniculatus.*

Short-term exposure of *L. japonicus* plants to salt stress (150 mM NaCl) did not affect the expression of *LjGPX* genes, except for *LjGPX4*, which was down-regulated between 1 and 24 h (Fig. 5). This result is at variance with the increase of mRNA levels observed for some *AtGPX* genes between 3 and 12 h of treatment with 500 mM NaCl (Sugimoto & Sakamoto, 1997; Rodríguez Milla *et al.*, 2003). This discrepancy can be attributed to the different plant species or, more likely, to variations in the growth and stress conditions used in the experiments. A transient increase of the *CsGPX1* mRNA level after 4-7 h of treatment with 200 mM NaCl was also observed in a salt-sensitive line, but not in a salt-tolerant line, of citrus cells (Avsian-Kretchmer *et al.*, 1999). Assuming a similar link between salt sensitivity and up-regulation of *GPX* genes, the failure of salt stress to induce expression of *LjGPX* genes suggests that *L. japonicus* plants are relatively salt tolerant and do not experience oxidative stress. This suggestion is supported by the finding that the same salt stress treatment neither affects expression of other antioxidant genes nor causes accumulation of lipid peroxides (data not shown).

In sharp contrast with salinity, the exposure of plants to SNP, a compound capable of releasing NO for at least 40 h (Bethke *et al.*, 2006), caused significant up-regulation of *LjGPX2* after 24 h, *LjGPX4* after 1, 3 and 24 h, and *LjGPX5* after 3, 6 and 24 h (Fig. 5). However, the most striking effect of SNP was, by far, on the expression of the *LjGPX6* gene. After only 1 h of SNP treatment, the steady-state mRNA level of *LjGPX6* increased by 30-fold and by 14-, 3- and 8-fold after 3, 6 and 24 h, respectively (Fig. 5). Because decomposition of SNP yields ferricyanide in addition to NO (Bethke *et al.*, 2006), a control treatment with potassium ferricyanide, at the same concentration as SNP, was included in the study. The only changes observed with ferricyanide were a decrease in the *LjGPX4* mRNA level after 24 h (Fig. 5), confirming that the effects of SNP were genuine to NO. So far, the effect of NO on GPX expression in plants had not been examined, but NO was found to up-regulate two other enzymes related to thiol metabolism, γ-glutamylcysteine synthetase and glutathione synthetase (Innocenti *et al.*, 2007). Our finding that NO triggers, within 1 h, the expression of specific *LjGPX* genes, in particular *LjGPX6*, strongly suggests an important role

of NO in the modulation of GPX function and also that at least some GPXs, in turn, may be part of signalling pathways downstream of NO.

The expression of *GPX* genes was examined in *L. corniculatus* plants treated with Cd or Al (Fig. 6), two metals with potent phytotoxic effects. A recent report has shown that, in addition to its general inhibitory effects on plant growth and photosynthesis, Cd induces oxidative stress, as evidenced by an increase in ROS production and in the amounts of oxidized lipids and proteins (Romero-Puertas *et al.*, 2004). In addition, two other pieces of evidence argue in favour of a role of GPX in the response to Cd. First, the protein level of an *A. thaliana* GPX is increased in response to Cd, and second, the resolution of the structure of poplar GPX5, in which thirty-two Cd atoms are bound to a dimer, suggest that at least some plant GPXs could act as a sink for Cd (Navrot *et al.*, 2006; Koh *et al.*, 2007). We found that Cd caused down-regulation of *GPX1*, *GPX2*, *GPX4* and *GPX6* between 3 and 24 h of treatment, and of *GPX5* only after 3 h (Fig. 6). In contrast, there was significant up-regulation (2.7-fold) of *GPX3*, *GPX4* and *GPX5* after 1 h of treatment and of *GPX3* after 24 h. Thus, Cd triggers a very rapid and transient activation (1 h) of specific *GPX* genes and a subsequent decrease (within 24 h) of the mRNA levels for all the genes (except *GPX3*) to control (or below control) values.

The effect of Al was also studied, for comparison, at the same concentration as Cd. The first symptom of Al toxicity, which develops at acid pH, is the inhibition of root growth (Barceló & Poschenrieder, 2002). Moreover, Al causes oxidative stress, an increase in antioxidant enzyme activities and lipid peroxidation (Sharma & Dubey, 2007; and references therein). We found that Al caused a general decrease of *GPX* transcripts after 6 and 24 h of treatment (Fig. 6). An earlier report showed that the treatment of *A. thaliana* plants with a combination of 100 μM Al and 100 μM Fe (pH 4.0) increased the *AtGPX6* mRNA level (Sugimoto & Sakamoto, 1997). However, this experiment is not comparable to ours due to major differences in the growth and treatment conditions of plants.

Immunoblot analyses and immunolocalization of GPX proteins

To gain further information on LjGPXs and also as a prerequisite for immunolocalization studies, we performed immunoblot analyses in leaves, roots and nodules of *L. japonicus* using an antibody raised against poplar GPX3.2 (Navrot *et al.*, 2006). This antibody was not isoform specific and recognized at least two LjGPX isoforms in the three plant organs (Supplementary Material Fig. S1). The apparent molecular masses of the immunoreactive proteins were 20.5 and 24 kDa, in the range expected for GPX proteins (Table 2). The antibody recognized also two immunoreactive bands in extracts from *L. corniculatus*, *A. thaliana*, and poplar (data not shown) and a single immunoreactive band of approximately 20.5 kDa when the antibody was challenged with poplar GPX3.2 (Supplementary Material Fig. S1). An antiserum raised against synthetic peptides based on the sequence of tomato GPXLe-1 (Herbette *et al.*, 2004), recognized the same two immunoreactive bands in leaf, root and nodule extracts of *L. japonicus*, further confirming that the two immunoreactive bands are genuine GPXs. No major changes were observed in the LjGPX protein levels of *L. japonicus* or *L. corniculatus* exposed to the stresses described above for up to 24 h (data not shown), although they were increased in plants treated with 150 mM NaCl for 7 d (Supplementary Material Fig. S1).

To our knowledge, there are only two reports on the subcellular localization of GPXs in plants. First, the antiserum against GPXLe-1 was used to detect the protein in the cytoplasm and cell wall of tomato internodes (Herbette *et al.*, 2004). Second, a fusion of the N-terminal extension of GPX3.2 with the green fluorescent protein was used to show that GPX3.2 is targeted to the mitochondria and chloroplasts of tobacco *(Nicotiana tabacum)* cells (Navrot *et al.*, 2006). We have used the GPX3.2 antibody, which was affinity-purified and hence more suitable for EM studies than the GPXLe-1 antiserum, to immunolocalize GPX proteins in *L. japonicus*. Plant material was high-pressure freezed for optimal preservation of antigenicity (Moran *et al.*, 2003). Most surprisingly, GPX was found exclusively in the chloroplasts or amyloplasts of leaves, roots and nodules (Fig. 7). Furthermore, the labelling

was associated with the starch grains in the uninfected cells of the cortex (Fig. 7a) and in the interstitial cells of the infected region (data not shown). The same localization of GPX was found in the proplastids of root tips (Fig. 7c) and in the chloroplasts of leaves (Fig. 7d). For comparison purposes, the localization of GPX was also examined in alfalfa, a crop legume with indeterminate nodules, and in *S. rostrata*, a tropical legume with determinate nodules on the roots and stems. Thus, abundant labelling was found in the starch grains in the infected cells of alfalfa nodules (Fig. 7e), as well as in the cortical chloroplasts of stem nodules (Fig. 7f) and in the leaf chloroplasts (Fig. 7g) of *S. rostrata*. Plastids in the non-photosynthetic root nodules on *S. rostrata* were also labelled (not shown). Negligible labelling was observed on sections incubated in non-immune serum, such as those of *L. japonicus* nodules (Fig.7b) and *S. rostrata* leaves (Fig. 7h).

The presence of GPX in association with starch grains had not been reported earlier but is consistent with the detection of thioredoxins *f* and *m* and of peroxiredoxin BAS1 in the amyloplasts (Balmer *et al.*, 2006; Barajas-López *et al.*, 2007). Thus, thioredoxins are substrates for both GPXs and peroxiredoxins, which are closely related enzymes (Navrot *et al.*, 2006). Our finding of GPX in the amyloplasts suggests that peroxides (their other substrates) are also formed in these organelles and points to a regulatory role of this enzyme, in connection with thioredoxins, in heterotrophic tissues. In this context, at least two questions are worthy of further research. First, the possibility that some GPX and peroxiredoxin isoforms are involved in the regulation of starch biosynthesis and/or mobilization, perhaps through changes in the thiol redox state mediated by ROS and/or RNS. Second, the inability of the antibody to detect any GPX in the cytosol or other subcellular compartments, despite that the *LjGPX3* gene, encoding a putative cytosolic or secretory protein (Table 2), is highly expressed at the mRNA level (Fig. 4). It is possible that *LjGPX3* is subjected to post-transcriptional regulation or that the localization of the corresponding protein is confined to certain plant tissues or cells. In any case, the results of the immunolocalization studies along with the surprisingly rapid and remarkable induction of specific *GPX* genes by NO underscore the complex regulation of the *GPX* genes in plants and strongly support the hypothesis that the GPX proteins play an important role not only as

antioxidants but also in stress or metabolic signalling.

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Supplementary Material

The following supplementary material is available for the article online:

Table S1 Primers used for quantification of *LjGPX* mRNA levels by qRT-PCR.

Table S2 Putative *cis*-acting regulatory elements of *LjGPX* promoters.

Fig. S1 Immunoblot analysis of LjGPX proteins in *L. japonicus*. *Upper panel*, Protein extracts from leaves (L), roots (R) and nodules (N) were blotted and challenged with an antibody against poplar GPX3.2. A positive control of purified GPX3.2 (C) was also included. *Lower panel*, Effect of short-term (1 d) and long-term (7 d) treatments with 150 mM NaCl on GPX expression in roots (R0 *versus* R150). The blots are representative of three replicates with extracts prepared from different plants. Lanes contained 10 ng (C) or 15 μg (all others) of protein. Molecular mass (kDa) of the immunoreactive proteins is indicated.

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(This link will take you to the article abstract.)

Legends for Figures

Fig. 1. Exon-intron organization of *LjGPX* genes. The UTRs are depicted in hatched boxes, ORFs in black boxes and introns in white boxes. Disrupted lines denote intron lengths not drawn to scale. All other lengths are drawn to scale.

Fig. 2. Amino acid sequences of LjGPX proteins. The threee conserved domains found in most animal and plant GPXs are marked in green, red and blue, respectively. The amino acid residues that form part of the proposed catalytic site of the GPXs are marked with asterisks. The cysteine residue marked with a green asterisk is replaced by selenocysteine in mammalian phosholipid hydroperoxide GPXs. Amino acid residues that are identical in at least five of the sequences are marked in white lettering on a black or colored background.

Fig. 3. Phylogenetic analysis of GPX proteins of vascular plants. Only complete sequences were considered. The tree was constructed by using the neighbor-joining method of CLUSTALW, with 1,000 bootstraps, and the bar indicates 0.1 substitution per site. Predicted localizations of proteins are denoted by different colors: green (chloroplasts), red (cytosol), blue (mitochondria), and brown (secretory pathway). Abbreviations of plant species: At, *Arabidopsis thaliana;* Ca, *Cicer arietinum*; Cs, *Citrus sinensis*; Gm, *Glycine max*; Ha, *Helianthus annuus*; Hv, *Hordeum vulgare*; Le, *Lycopersicon esculentum*; Lj, *Lotus japonicus;* Mc, *Mesembryantemum crystallinum*; Md, *Malus* x *domestica*; Mt, *Medicago truncatula;* Ns, *Nicotiana sylvestris*; Os, *Oryza sativa;* Pt, *Populus trichocarpa*; Ps, *Pisum sativum*; So, *Spinacia oleracea*; Tm, *Triticum monococcum*; Vv, *Vitis vinifera*; Zm, *Zea mays*. Accession numbers for *L. japonicus* genes are indicated in Table 1 and those of other plant species are as follows (in brackets): At1 (At2g25080), At2 (At2g31570), At3 (At2g43350), At4 (At2g48150), At5 (At3g63080), At6 (At4g11600), At7 (A4g31870), At8 (At1g63460), Ca (CAD31839), Cs (CAA47018), Gm1 (TC203397), Gm2 (TC203326), Gm3 (TC203271), Ha1 (CAA74775), Ha2 (CAA75009), Hv1 (CAB59895), Hv2 (CAB59893), Hv3 (CAB59894), Hv4 (BAC55016), Le1 (CAA75054), Le2 (AAP59427), Mc (CAB96145), Md (AAQ03092),

Ns (CAA42780), Mt1 (TC94412), Mt2 (BM814275), Mt3 (TC106328), Mt4 (TC94581), Os1 (Os04g0556300), Os2 (Os06g0185900), Os3 (Os02g0664000), Os4 (Os03g0358100), Os5 (Os11g0284900), Ps (CAA04142), Pt1 (ABK96776), Pt2 (DT518382), Pt3.1 (ABK96047), Pt3.2 (ABK94488), Pt4 (ABK95195), Pt5 (2P5Q_A), So (BAA22194), Tm (AAQ64633), Vv (CB978870); Zm (AAM88847).

Fig. 4. Expression of *LjGPX* genes in leaves, roots and nodules of *L. japonicus*. (a) Relative expression of each gene in the three plant organs. Steady-state mRNA levels were normalized with respect to *ubiquitin* and were expressed relative to the values of the *LjGPX6* gene, which were given an arbitrary value of 1. (b) Relative expression of each gene in leaves and nodules with respect to roots. Steady-state mRNA levels were normalized with respect to *ubiquitin* and were expressed relative to the values found in the roots, which were given an arbitrary value of 1. All data are means ± SE of six replicates.

Fig. 5. Time-course expression analysis of *LjGPX* genes in roots of *L. japonicus* exposed to NaCl (150 mM), SNP (100 μM) or potassium ferricyanide (100 μM). Steady-state mRNA levels were normalized with respect to *ubiquitin* and expressed relative to the values at time 0 (control), which were given an arbitrary value of 1. Data are means ± SE of three to six replicates.

Fig. 6. Time-course expression analysis of *GPX* genes in roots of *L. corniculatus* exposed to 20 μM Cd or 20 μM Al. Steady-state mRNA levels were normalized with respect to *ubiquitin* and expressed relative to the values at time 0 (control), which were given an arbitrary value of 1. Data are means ± SE of three to six replicates.

Fig. 7. Immunogold localization of GPX proteins within plastids of leaves, roots and nodules of legumes. (a) Plastid (p) within a cortical cell in a nodule of *L. japonicus*. The immunogold labelling (arrow) is specifically localized on the starch grain (s) in the plastid. (b) Serial section to (a) but treated with non-immune serum substituted for the GPX antibody. There is no significant labelling on the starch grain (s) or elsewhere in the cortical cell.

(c) Immunogold labelled (arrow) starch grain (s) in a plastid (p) within a *L. japonicus* root tip cell. Note that the adjacent mitochondria (m) are not labelled. (d) Immunogold labelling (arrow) of starch grains (s) in a leaf chloroplast of *L. japonicus*. (e) Immunogold labelling (arrows) of starch (s) in a plastid within an infected cell of an alfalfa nodule. (f) Chloroplast (c) in a *S. rostrata* stem nodule with immunogold labelling (arrows) of the starch grains (s) within it. (g) Immunogold labelling of starch grains (s) within chloroplasts (c) in a leaf of *S. rostrata*. (h) Serial section to (g) but treated with non-immune serum substituted for the GPX antibody. There is no significant labelling on the starch grain (s) or elsewhere in the leaf cell. b, bacteroid; c, chloroplast; is, intercellular space; p, plastid; v, vacuole. Bars, 1 μm.